General and Comparative Endocrinology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

# Growth hormone promotes synaptogenesis and protects neuroretinal dendrites against kainic acid (KA) induced damage

Thomas Fleming [a,1], Carlos G. Martinez-Moreno [b,1], Martha Carranza [b], Maricela Luna [b], Steve Harvey [a], Carlos Arámburo [b,*]

[a] Department of Physiology, University of Alberta, Edmonton T6G 2H7, Canada
[b] Departamento de Neurobiología Celular y Molecular, Instituto de Neurobiología, Campus Juriquilla, Universidad Nacional Autónoma de México, Querétaro, Qro 76230, Mexico

ARTICLE INFO

*Article history:*
Received 26 November 2017
Revised 7 February 2018
Accepted 14 February 2018
Available online xxxx

*Keywords:*
Growth hormone
Synaptogenesis
Neuroprotection
Neuroretina
Kainate
Neuroregeneration

ABSTRACT

There is increasing evidence that suggests a possible role for GH in retinal development and synaptogenesis. While our previous studies have focused largely on embryonic retinal ganglion cells (RGCs), our current study demonstrates that GH has a synaptogenic effect in retinal primary cell cultures, increasing the abundance of both pre- (SNAP25) and post- (PSD95) synaptic proteins. In the neonatal chick, kainate (KA) treatment was found to damage retinal synapses and abrogate GH expression. In response to damage, an increase in Cy3-GH internalization into RGCs was observed when administered shortly before or after damage. This increase in internalization also correlated with increase in PSD95 expression, suggesting a neuroprotective effect on the dendritic trees of RGCs and the inner plexiform layer (IPL). In addition, we observed the presence of PSD95 positive Müller glia, which may suggest GH is having a neuroregenerative effect in the kainate-damaged retina. This work puts forth further evidence that GH acts as a synaptogenic modulator in the chick retina and opens a new possibility for the use of GH in retinal regeneration research.



## 1. Introduction

Growth hormone (GH) is produced in the pituitary and has many documented effects throughout the body, particularly effects on cell differentiation, proliferation, and survival (Oberbauer, 2015). However, in addition to its production in the pituitary, GH is now known to have many extra-pituitary sites of production (Harvey, 2010). Of particular importance is its production in the neuroretina (NR), where it is expressed together with GH receptor (GHR), suggesting a local autocrine/paracrine mode of action (Harvey et al., 2015).

Several studies from our group have suggested critical roles for extrapituitary GH in retinal development and function (Harvey et al., 2007). For instance, GH involvement in axon growth and synaptogenesis has been demonstrated in immunopanned retinal ganglion cells (RGCs), which express both GH and its receptor as well as respond to exogenous GH by increasing axon length, whereas knockdown of endogenous GH expression with small interfering RNA (siRNA) resulted in a blockage of long axon formation (Baudet et al., 2009). Additionally, exogenous GH treatments were able to increase GAP43 immunoreactivity in QNR/D cells (a quail RGC line) and SNAP25 immunoreactivity in chicken neuroretinal explants *in vitro* as well as increase SNAP25 immunoreactivity *in vivo* after the injection of exogenous GH into the vitreous of ED10 chick embryos (Fleming et al., 2016). Extrapituitary GH is also neuroprotective in the chick neuroretina where its local expression is anti-apoptotic during waves of apoptosis that occur during normal development of the chick retina (Sanders et al., 2008, 2011). Furthermore, GH has shown to be neuroprotective against glutamate/BSO induced excitotoxicity *in vitro* in QNR/D cells (Martínez-Moreno et al., 2016).

Recent evidence in the chicken and green iguana suggests GH is a neuroprotective factor against injury caused by kainic acid (KA), a glutamatergic agonist (Ávila-Mendoza et al., 2016; Martínez-Moreno et al., 2018). In the green iguana, locally produced GH was shown to be up-regulated in response to KA treatment and exogenous GH was able to prevent cell loss in the inner nuclear layer (INL) and ganglion cell layer (GCL) caused by KA injection (Ávila-Mendoza et al., 2016). Similar results were found in the chick, where the neuroprotective effects of GH were mainly located in the INL and inner plexiform layer (IPL) (Martínez-Moreno et al., 2018). While these results show that GH is able to protect the

* Corresponding author.
*E-mail address:* aramburo@unam.mx (C. Arámburo).
[1] T.F. and C.G.M.M. contributed equally to this work

https://doi.org/10.1016/j.ygcen.2018.02.011
0016-6480/

cytoarchitecture of the retina after excitotoxic damage, its ability to protect/regenerate retinal synapses after KA treatment have not been previously reported.

The objective of this study was to determine if GH acts as a synaptogenic modulator by examining its protective effect on synapses of the retina under experimentally induced excitotoxicity with KA. The work provides further evidence showing that GH promotes synaptogenesis *in vitro* and also protects dendrites in the neuroretina after excitotoxic insult.

## 2. Materials and methods

### 2.1. Animals

Pathogen-free, fertilized eggs (*Gallus gallus*, White Leghorn) eggs were obtained from the University of Alberta Poultry research center (Edmonton, Canada) for use in primary cell culture experiments. Eggs for post-hatch experiments were obtained from Pilgrim's Pride (Querétaro, México). Eggs were incubated at 38 °C in a humidified air chamber and were rotated one-quarter of a revolution every 50 min during incubation. Chicken embryos and post-hatch chicks were sacrificed following protocols approved by the University of Alberta animal welfare committee and by the Instituto de Neurobiología (UNAM) bioethical committee.

### 2.2. Primary retinal cell culture

Chicken embryos (ED10) were anesthetized in ice for 5 min and then sacrificed by decapitation. Eyes were enucleated, the vitreous was removed and neuroretinas were micro-dissected. Using a stereoscopic microscope, pigment epithelium contamination was removed. For cell disaggregation, the neuroretinas were incubated in 70 U/ml collagenase (Sigma, Saint Louis, MO, USA) in DMEM (Gibco, Grand Island, NY, USA) for 40 min at 37 °C. The cells were vortexed and mechanically triturated with a pipette every 10 min to aid in disaggregation. Cells were then pelleted and washed with DMEM + 10% FBS (Gibco, USA) twice. Cells were resuspended in DMEM + 10% FBS (Gibco, USA) and plated on 35 × 10 mm poly-L-lysine coated (Sigma, Saint Louis, MO, USA) plates (Corning, New York, USA). Cells ($5.5 \times 10^5$) were counted using trypan blue and hemocytometer, and plated in 24-well plates. Cell cultures were stabilized in a humidified incubator at 39 °C and 5% $CO_2$.

### 2.3. Treatments

For *in vitro* primary cell culture experiments, recombinant chicken growth hormone (rcGH) (Rehovot, PRL, Israel) was dissolved in bicarbonate buffer (pH 9.0) and administered in DMEM with 10% FBS (Gibco, Grand Island, NY, USA) at a final concentration of 1 (PSD95) or 10 (SNAP25) nM for 24 h. Negative controls were incubated without rcGH. For Cy3-GH experiments, 300 ng of Cy3-GH was injected either 1 h before KA injection (20 μg) or 1 or 24 h after and incubated for 24 h. Cy3-GH pre-incubated with a GH antibody at room temperature for 24 h was used for control. Cy3 conjugation to GH was performed according to Cy3 Mono-Reactive dye pack manufacturer's instructions (GE Healthcare Life Sciences, Amersham, ON, Canada). For intravitreal injections, KA (20 μg) and rcGH (300 ng and 150 ng) were diluted in injectable water (re-suspended prior to injections). *In vivo* injections for analysis by Western blot and qPCR were administered into the left eye using the following strategy: a prior GH dose (300 ng, 24 h before damage), simultaneous KA (20 μg) + GH (300 ng), and two injections at 24 h and 48 h post-injury (GH, 300 ng). Eyes were stabilized for 24 h and were then collected. Treatments for eyes analyzed by IHC were injected as follows: GH (300 ng) 24 h prior, GH (150 ng) and KA, and GH (300 ng) 24 h after after-damage. The opposite eye (right) was injected with vehicle as a negative control. Pre-and post-injury GH injections were used according to Fischer and Reh (2002), Todd et al. (2015, 2016), and Ritchey et al. (2012) for the induction of growth factor expression and neuroprotection in the avian retina.

### 2.4. Immunohistochemistry

For immunohistochemical analysis, eyes were fixed with Davidson fixative for 48 h, dehydrated in ethanol, and embedded in paraffin. Sections (5 μm) were cut with a microtome and mounted on pretreated glass slides (Fisher Scientific, Hampton, NH, USA). Paraffin was removed from the slides with Citrisolv (Fisher Scientific, Hampton, NH, USA). Sections were gradually rehydrated in absolute alcohol (100%), ethanol (95, 70, 50 and 30%), distilled water, then lastly samples were equilibrated in PBS for 1 h. Tissues were then boiled in citrate buffer (10 nM sodium citrate, 0.05% Tween 20; pH 6.0) for 20 min to unmask epitopes. PSD95 and GAP43 antibodies were both used at a dilution of 1:500 in TPBS with 1% non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Negative controls were performed in the absence of primary antibody. After washing (3 × 10 min) in TPBS, sections were incubated for 2 h with Alexa Fluor 488 goat anti-mouse (GAP43; Invitrogen Molecular Probes, Eugene, Oregon, USA) or Alexa Fluor 594 donkey anti-rabbit (PSD95; Invitrogen, USA) IgG antibodies at a concentration of 1:2000 in TPBS with 1% non-fat dry milk (Bio-Rad). After washing in TPBS (3 × 10 min), the sections were stained with DAPI and mounted with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA).

### 2.5. Cryosectioning

Cy3-GH treated eyes were extracted and soaked for 24 h in a 30% sucrose solution. After 24 h, the cornea and vitreous were removed and allowed to incubate for an additional 24 h. Eyes were then fixated in a 4% PFA solution for 24 h. Eyes were then cryosectioned into 5 μm sections with a microtome, stained for DAPI and visualized using a confocal microscope Zeiss Axiovert 200 LSM 510.

### 2.6. Western blot analysis

Total protein extracts from primary cell cultures and whole chicken NRs were extracted by sonication and homogenization in presence of protease-inhibitor cocktail (Mini-complete, Roche Diagnostics, Basel, Switzerland) in 50 mM Tris-HCl buffer (pH 8.0). Samples were boiled for 5 min with 2× sample buffer containing bromophenol blue and 5% β-mercaptoethanol. Equivalent amounts of protein were added to each lane (40 μg) and electrophoresed in a 12.5% polyacrylamide gel using the buffer system of Laemmli (1970). The Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) was used to ensure equal protein loading of each lane. Protein samples were transferred from the gel onto a nitrocellulose membrane. For immunoblotting, membrane blocking was carried out using 5% non-fat milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in PBS for 1 h. Primary antibodies solutions (Table 1) were made in a 1% non-fat milk TPBS solution, added to membranes and were incubated overnight at 4 °C with moderate shaking. Secondary antibody solutions were made at a concentration of 1:5000 with IgG-HRP cat anti-goat (Santa Cruz Biotechnology, CA, USA), goat anti-rabbit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), or goat anti-mouse (Bio-rad) in a 1% non-fat milk/TPBS solution, and membranes were incubated for 2 h at RT. Immunoreactive bands were developed by chemoluminescense using ECL reagent (Amersham Biosciences, Montreal, Canada) after exposing the membranes to Kodak Biomax ML films which were then pro-

Please cite this article in press as: Fleming, T., et al. Growth hormone promotes synaptogenesis and protects neuroretinal dendrites against kainic acid (KA) induced damage. Gen. Comp. Endocrinol. (2018), https://doi.org/10.1016/j.ygcen.2018.02.011